Analysis of Tooth Innervation in Microfluidic Coculture Devices

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Abstract: Innervation plays a key role in the development, homeostasis, and regeneration of organs and tissues. However, the mechanisms underlying these phenomena are not well understood yet. In particular, the role of innervation in tooth development and regeneration is neglected. Cocultures constitute a valuable method to investigate and manipulate the interactions between nerve fibers and teeth in a controlled and isolated environment. Microfluidic systems for allow cocultures of neurons and different cell types in their appropriate culture media, while permitting the passage of axons from one compartment to the other. Here we describe how to isolate and coculture developing trigeminal ganglia and tooth germs in a microfluidic coculture system. This protocol describes a simple and flexible way to coculture ganglia/nerves and their target tissues and to study the roles of specific molecules on such interactions in a controlled and isolated environment.

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Analysis of tooth innervation in microfluidic co-culture devices

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Running Head: Co-culture in a microfluidic system

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Summary

Innervation plays a key role in the development, homeostasis and regeneration of organs and tissues. However, the mechanisms underlying these phenomena are not well understood yet. In particular, the role of innervation in tooth development and regeneration is neglected. Co-cultures constitute a valuable method to investigate and manipulate the interactions between nerve fibres and teeth in a controlled and isolated environment. Microfluidic systems allow co-cultures of neurons and different cell types in their appropriate culture media, while permitting the passage of axons from one compartment to the other. Here we describe how to isolate and co-culture developing trigeminal ganglia and tooth germs in a microfluidic co-culture system. This protocol describes a simple and flexible way to co-culture ganglia/nerves and their target tissues and to study the roles of specific molecules on such interactions in a controlled and isolated environment.

1. Introduction

Innervation plays a key role in the development, homeostasis and regeneration of organs and tissues. Furthermore, innervation is involved in the regulation of stem cell proliferation, mobilization and differentiation [1–4]. In spite of the rich innervation of adult teeth, and in contrast to all other organs and tissues of the body, developing teeth start to be innervated at the earliest postnatal stages [5, 6]. Sensory nerves from the trigeminal ganglia and sympathetic nerves from the superior cervical ganglia innervate the adult teeth [5, 6]. Teeth develop as a result of sequential and reciprocal interactions between the oral ectoderm and cranial neural crest-derived mesenchyme. These interactions give rise to epithelial-derived ameloblasts and mesenchyme-derived odontoblasts that are responsible for the formation of enamel and dentin, respectively [7]. During embryogenesis, nerve fibres projecting from the trigeminal ganglia progressively surround the developing tooth germs but they do not contact them neither penetrate them. Nerve fibres enter the dental pulp mesenchyme at more advanced developmental stages that are associated with odontoblast differentiation and dentin matrix deposition

events [8]. Dental pulp innervation is completed soon after tooth eruption in the oral cavity [6]. Different studies investigated the role of innervation in tooth development [1, 9–13]. Nevertheless, the role of innervation in tooth formation and regeneration is still highly controversial in mammals [1].

Co-cultures constitute a valuable method to investigate and manipulate the interactions between nerve fibres and teeth in a controlled and isolated environment [14–17]. At the same time, co-culturing involves important technical adjustments. For example, nerves and specific dental tissues (e.g. dental pulp, dental follicle, dental epithelium) often require different culture media in order to guarantee survival and physiological behaviour of the tissues for long periods of time [18].

Microfluidics systems allow co-cultures of neurons and different cell types in their appropriate culture media. In these devices, dental tissues and neurons are separated in different compartments, while allowing the growth of axons from the neural cell bodies through microchannels towards the compartment containing their target tissue [18–20].

We have recently demonstrated that trigeminal ganglia (TG) and teeth are able to survive for long periods of time when co-cultured in microfluidic devices [18]. Moreover, we have demonstrated that teeth from different developmental stages maintain in these *in vitro* conditions the same repulsive or attractive effects on trigeminal innervation that they show *in vivo* [18]. This protocol provides information about a simple, powerful and flexible way to co-culture ganglia/nerves and target tissues and to study the roles of specific molecules on such interactions in a controlled and isolated environment.

2. Materials

2.1 Material for dissection

1. Dissection scissors and forceps, dissection needles (insulin needles).

2. Dissection glass dish

3. Stereoscope

2.2 Solutions

1. Phosphate-Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

2. Poly-D-lysine: stock 1 mg/ml, dilute to 0.1 mg/ml in distilled, sterile H_2O to obtain the working solution.

3. Laminin: stock 100µg/ml (Sigma-Aldrich, L2020), dilute to 5 µg/ml in neurobasal medium to obtain the working solution.

4. Paraformaldehyde 4% in PBS (PFA 4%).

2.2 Culture media

1. Tooth culture medium: DMEM-F12, 20% Foetal Bovine Serum, 10 U/ml penicillin/streptomycin, 2mM L-glutamine, 150 µg/ml ascorbic acid.

2. Trigeminal ganglia culture medium: Neurobasal medium, B27, 50 ng/ml recombinant nerve growth factor (β-NGF), 10 U/ml penicillin/streptomycin, 2mM L-Glutamine.

2.3 Microfluidic co-culture chambers components

3. AXIS Axon Isolation Device: Millipore, AX15010-TC (microchannels of different length are available).

4. Glass coverslips 24 mm X 24 mm.

5. 6-wells plate or 35 mm Petri Dish.

6. Biopsy punch (diameter: 1-4 mm).

7. Vacuum chamber and pump.

3. Methods

3.1. Preparation of dissection material, culture media, microfluidic devices

1. Autoclave micro-dissection forceps and scissors (121°C, sterilization time: 20 minutes) and store them in a sterile container.

2. Sterilize glass coverslips (24 mm x 24 mm) by incubating them in 1M HCl for 24 hours at 37°C. Wash them three times with sterile, distilled H_2O and three times with ethanol 99%. Dry then the coverslips under sterile flow hood. Finally, autoclave the coverslips to complete the sterilization. Coverslips can be stored in ethanol 70%.

3. Remove carefully the AXIS Axon Isolation Devices from the package using sterile forceps and place them in a sterile Petri dish.

4. Using a sterile biopsy punch, create one hole per sample to be cultured in correspondence of the culture chambers (figure 1; see Note 1).

5. Sterilize the AXIS Axon Isolation Devices by immerging them in ethanol 70%. Dry then AXIS Axon Isolation Devices and coverslips completely under a sterile flow hood. Wait a minimum of 3 hours before proceeding (see Note 2).

6. Place each coverslip into a 35mm Petri dish or into a well within a 6-wells plate (see Note 3).

7. Place the AXIS Axon Isolation Device onto the coverslip and press gently but firmly with a forceps with bent ends in order to allow full adhesion between the isolation device and the glass coverslip (figure 1).

8. In each culture chamber, pipette 150µl of poly-D-lysine (0.1 mg/ml). Place the microfluidic devices under vacuum for five minutes, in order to remove all the air from the culture chambers (see Note 4). Incubate the devices with poly-D-lysine overnight at 37°C.

11. Wash chambers three time with sterile, distilled H_2O. Fill chambers with 150 µl laminin working solution (5 µg/ml) and incubate for 2 hours at 37°C (see Note 5).

13. Prepare 10 ml of medium for trigeminal ganglia cultures and 10 ml for tooth organ cultures.

3.2 Mouse embryo generation and dissection

Animal treatments have to be performed in accordance with the Animal Welfare Law and with the regulations of the deputed institution.

1. Determine embryonic age according to vaginal plug (vaginal plug: embryonic day of development 0.5: E0.5) and confirm it via morphological criteria. For this protocol we generally use E14.5 – E17.5 mouse embryos.

2. Clean the dissection area and the stereoscope with ethanol 70%.

3. Sacrifice the pregnant mother via cervical dislocation. Block the neck of the mouse with the first and second finger onto a grid and pull with decision the tail.

4. Dissect the skin around the lower abdomen and open the abdomen using scissors. Locate the uterus: during such late stages of pregnancy, the uterus fills the abdominal cavity and the embryos are clearly visible.

5. Dissect out the uterus and place in a tube filled with PBS on ice. When on ice, the tissue can be left for several hours. Discard the corpse of the mother according to the guidelines of your institution.

6. Dissect out the embryos from the uterus and free them from their extraembryonic tissues. Place the embryos in PBS on ice.

7. Decapitate the embryos using scissors and separate the lower jaw from the rest of the head using micro-dissection scissors. Remove precisely the lower jaw without damaging the trigeminal ganglia (see Note 6). Preserve the lower jaw and the rest of the head in cold PBS on ice.

8. To dissect TG, take the head and place it onto a dissection glass Petri dish, previously filled with cold PBS. Using the forceps remove the skin and the skull. Remove then the telencephalon and the cerebellum by placing forceps below the telencephalon and lift. The telencephalon and the cerebellum will flip together leaving the bottom of the skull exposed.

9. Localize the trigeminal ganglia. Use the forceps to separate the TG from the trigeminal nerves. Eliminate the remnants of the trigeminal projections using the dissection needles as knifes. Place the dissected TG in a Petri dish filled with cold PBS and keep them on ice.

10. To dissect embryonic teeth, place the lower jaw, previously separated from the skull onto a dissection glass Petri dish filled with cold PBS. Remove using dissection needles as knifes the tongue and the skin surrounding the jaw. Separate the left and the right hemi-jaws by cutting along the midline of the jaw. The tooth germs are easily visible. Isolate the tooth germs using dissection needles and remove the excess of non-dental tissues. Place the dissected tooth germs in a Petri dish filled with cold PBS and keep them on ice (figure 1).

3.3 Microfluidic co-cultures

1. After dissection remove laminin from the microfluidic devices. Fill the chambers with 200 µl of the respective media.

2. Transfer gently with forceps the dissected TG and tooth germs into the holes created by punching (figure 1, see Note 7). Culture the samples in incubator at 37°C with 5% CO_2. Change the culture medium every 48 hours (see Note 8).

3. Co-cultures can be easily imaged by time-lapse microscopy during the culture period by placing the culture dishes in an environmental chamber (37°C, 5% CO2). Focus on the extending neurites in order to follow the progress of innervation. Co-cultures can be maintained for over 10 days.

4. After the culture period different analysis can be performed (see note 9). In order to fix the cultures wash the chambers by pipetting 150µl of PBS into one well per chamber and letting PBS flow through

the chambers three times. Remove the PBS and fix the samples by pipetting 150µl of PFA 4% in one well per chamber. Incubate the samples at room temperature for 15 minutes. Wash the chambers twice with PBS as described above.

4. Notes

1. Do not perform the punch too close (< 0.5 mm of distance) to the microchannels as they might be damaged by the pressure applied.

2. Both the AXIS Axonal Isolation Device and the glass coverslip must be completely dry before the assembly of the microfluidic device. Incomplete drying leads to incomplete adhesion of the device to the underlying coverslip, which in turn results into abundant leakage of the microchannels and loss of the isolation of the chambers.

3. If stability of the device is needed, a small drop of PBS or water can be placed on the dish before placing the coverslip onto it. However, this procedure will also lead to increased difficulties in detaching the coverslip at the end of the culture period.

4. If after the vacuum treatment air bubbles can still be seen within the culture chambers, remove all the poly-D-lysine solution and pipette it with force directly into the chambers. Air must be removed from the culture chambers: if air bubbles are present they will not be filled with medium at any point during the culture and they will therefore prevent growth of any cell/axon in that region of the device.

5. It is convenient to perform the laminin coating over the dissection time. The laminin coating can be also performed for periods longer than 2 hours without consequences.

6. The trigeminal ganglion is localized in close proximity to the temporomandibular joint. Therefore, the risk of damaging the trigeminal ganglion while dissecting the lower jaw is high. Carefully cut the posterior end of the lower jaw bending the dissection scissors away from the skull.

7. Particular care needs to be taken when transferring the organs into the microfluidic devices. Embryonic organs are very light and they therefore tend to float. Once you have transferred the organs to the device ensure that they sink until they contact the bottom.

8. Do not empty the chambers completely and do not pipette directly into the culture chambers. Complete emptying of chambers would result in the formation of air bubbles within the chambers. Direct pipetting into the chamber would result in axonal damage. To avoid these issues, remove the medium pointing the pipette towards the external side of the wells. Similarly, pipette the fresh medium on the side of the wells located opposite to the culture chambers.

9. We have analysed the co-cultures via immunofluorescence and subsequent widefield and confocal microscopy. Immunostainings can be easily performed directly in the microfluidic devices by pipetting the different solutions in the wells, as done for the medium during the culture period. For the subsequent imaging the whole device can be detached from the well/dish by gently lifting the coverslip from the underlying plastic using forceps. This procedure is generally safe. However, if the coverslip is sealed by a drop of PBS or water to the underlying plastic (as indicated in note 5), the risk of damaging the coverslip by lifting it is high. To avoid this issue some PBS or water can be pipetted at the borders of the coverslip: the liquid will detach the coverslip from the well/dish. Other analyses can be performed onto these cultures such as gene expression analysis [19].

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Figures and figures captions

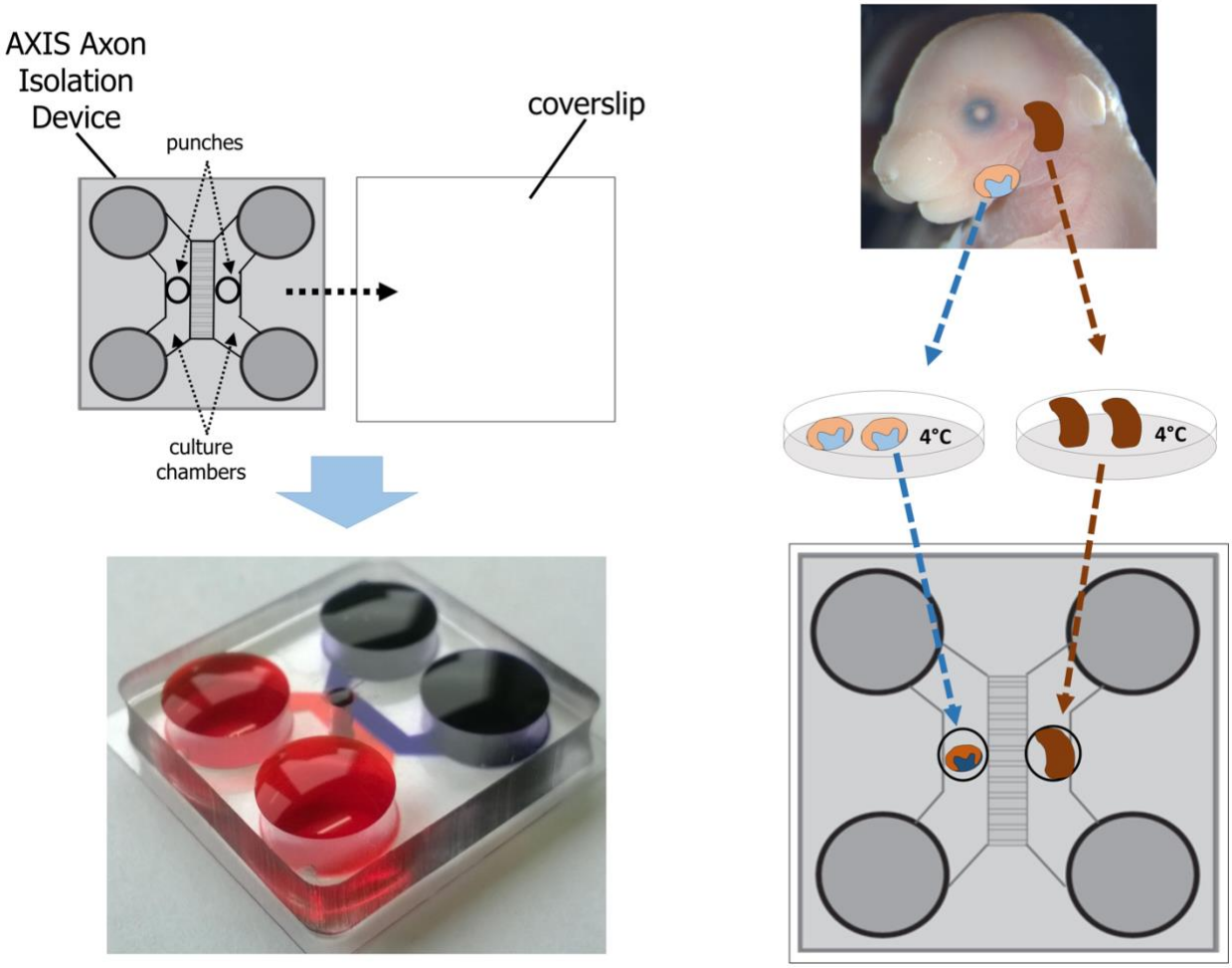

Figure 1. Schematic representation of the protocol. Left: structure and main components of the microfluidic co-culture device. Right: localization of trigeminal ganglia and tooth germs in the mouse embryo head and their placement in the microfluidic co-culture device.